A PERSISTENT DIURNAL RHYTHM OF LUMINESCENCE IN GONYAULAX POLYEDRA[1]

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The photosynthetic marine dinoflagellate, *Gonyaulax polyedra*, emits a brief flash of light (duration, about 90 milliseconds) when stimulated by agitation. It is one of the many organisms responsible for the luminescent display sometimes observed in the ocean at night when the water is disturbed (see Harvey, 1952). Previous studies with this organism (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a) have shown that the luminescent response to stimulation varies rhythmically in a diurnal fashion. Cultures grown in natural illumination, or in artificial lights with alternating light and dark periods of 12 hours each (= LD), display a much greater luminescence during the dark period (Fig. 2).

When LD cultures are transferred to a dark chamber, the rhythm continues but its amplitude decreases progressively. By action spectra studies, it has been found (Sweeney, Haxo and Hastings, unpublished data) that this decrease in amplitude arises from the need for light in the organic nutrition of *Gonyaulax*, via photosynthesis. This finding prompted the search for constant environmental conditions under which the endogenous rhythm would persist, without the loss of amplitude which occurs in continuous darkness.

The possibility of maintaining the cells heterotrophically was explored, but the consistently negative results obtained indicated that *Gonyaulax* is an obligate photo-auxotroph. Continuous bright light inhibits the rhythmic fluctuations in luminescence, and it has not been possible to separate, by using light of different colors, the photosynthetic requirements for light from the inhibitory action of light on rhythmicity. It has been found, however, that if LD cultures are placed in a continuous dim light, the rhythm of luminescence persists without loss of amplitude. It has thus been possible to investigate in some detail the nature of this endogenous rhythm.

Materials and Methods

G. polyedra has been maintained in a modified sea water medium described previously (Sweeney and Hastings, 1957a). The growth rate is dependent upon light, temperature, and the concentrations of mineral nutrients. The maximum growth rate which we have measured is one division per day, but under the condi-

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tions used in the experiments to be described the rates were always less than this. The illumination was provided by "cool white" fluorescent lamps, the intensity being measured in foot-candles with a Weston illumination meter.

The experimental procedure was as follows: stock cultures were maintained in Fernbach flasks containing 1500 ml. of medium. In preparation for an experiment, 2-ml. aliquots from these cultures were pipetted into each of several hundred test tubes at cell densities between 2000 and 7500 cells per ml. All tubes were then subjected to the appropriate conditions of light and temperature. To measure the luminescence at any given time, two tubes were removed, assayed, and then discarded. The cells were stimulated to luminesce by bubbling air through the cell suspension, and the resulting phototube current was accumulated on a capacitor. Luminescence is expressed in terms of the total amount of light emitted during one minute of stimulation, at the end of which time essentially all luminescence has ceased. Additional details of the light measurement procedure may be found elsewhere (Sweeney and Hastings, 1957a).

Results

Demonstration of the persistent rhythm. A persistent rhythm of luminescence may be observed if cells which have been kept for a time under LD conditions are transferred to continuous dim light (about 100 foot-candles). A typical example of the persistent rhythm under conditions of constant light and constant temperature is shown in Figure 5. In similar experiments, we have continued measurements for as long as 14 days; the rhythmic pattern continues undamped during this time. At the light intensity used in such experiments there was little growth.

The natural period of the rhythm. The period of the rhythm is measured by the time between successive maxima in luminescence. When the cells are subjected to alternating light and dark periods on a daily (24-hour) schedule, the period of the rhythm is 24 hours (Fig. 2). Under conditions of constant illumination, however, the rhythmic changes have a period which is close to, but not necessarily exactly 24 hours. Pittendrigh and Bruce (1957) have referred to this as the *natural period,* or the innate period of an endogenous rhythm when light and temperature are held constant.

The natural period in *Gonyaulax* is a function of at least two environmental factors, light intensity and temperature. The effect of light intensity upon the period is illustrated in Figure 1. Cells were placed in continuous light at three different intensities, and it is evident that the natural period was shorter at higher intensities. These experiments also illustrate the light intensity dependence of the inhibitory effect of continuous illumination upon the rhythm. At the two higher light intensities the amplitude of the rhythm was progressively damped, while at the lowest light intensity no marked damping of the amplitude of the rhythm was evident.

The effect of temperature upon the natural period is not large but, contrary to expectation, the period becomes longer rather than shorter as the temperature is raised (Hastings and Sweeney, 1957b). At 16° C. the period was found to be 22.8 hours while at 26.7° C. it was 26.5 hours. A Q_{10} of less than 1.0 is unusual, and the results were interpreted as evidence for a compensation mechanism which functions to keep the period approximately temperature-independent.

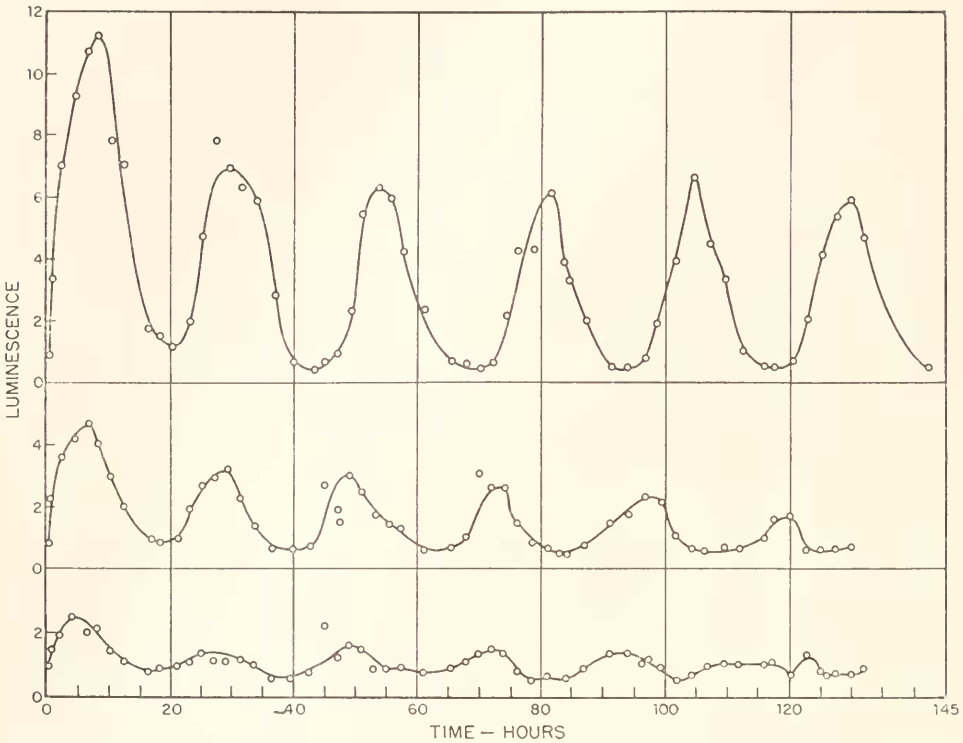

FIGURE 1. The effect of light intensity upon the natural period at constant temperature (21° C.). The cells were grown in LD conditions (800 foot-candles during the light period). The beginning of the experiment, shown on the graph as 0 time, fell at the end of a normal light period. At this time, some cells were placed in the dark, and others in light of 120 foot-candles (upper curve), 380 foot-candles (middle) and 680 foot-candles (bottom). The average periods were as follows: 680 foot-candles, 22.0 hours; 380 foot-candles, 22.8 hours; 120 foot-candles, 24.5 hours; dark, 24.5 hours (not shown on graph; one period measured).

In view of the relatively small temperature effect, the period of this rhythm may be characterized as essentially temperature-independent.

The endogenous nature of the diurnal rhythm. The persistence of the rhythm of luminescence under conditions of constant temperature and light intensity indicates that the mechanism of the rhythmicity is endogenous. Several other experiments serve to support this conclusion.

Figure 2 illustrates one of many experiments in which the phase of the rhythm was shifted by changing the time at which the light and dark periods occurred. In such experiments the phase (*i.e.,* the solar time at which the maximum in luminescence occurs) may be shifted so that it will bear any desired relationship to the solar day. In cultures which are subsequently transferred to constant conditions of dim light or darkness, the phase of the persistent rhythm is related to the previous light and dark program rather than to solar time, or any other factor. Changes in the phase of the endogenous rhythm have not been observed when light and temperatures were held constant.

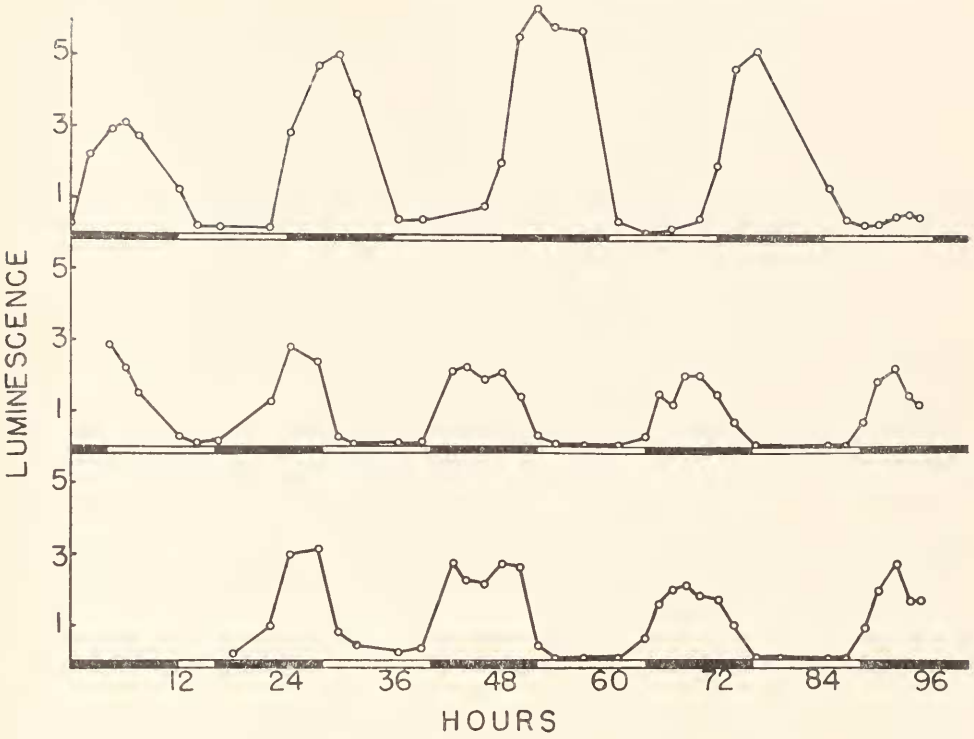

FIGURE 2. This experiment illustrates the effect of changing the solar time at which the light and dark periods occur. The upper curve shows the pattern of luminescence changes in an LD culture which had been on the schedule indicated for some time. The black bars on the time axis indicate dark periods. The lower two graphs illustrate the effect of imposing upon cultures (which were previously on the schedule shown in the top graph) an LD schedule in which the light and dark periods were at a different time of day. The new schedules were started at zero hours on the graph. Temperature, about 26° C. Light intensities used, about 250 foot-candles.

A series of experiments has been carried out from which it is evident that pre-treatment with diurnal light and dark periods (*i.e.*, one dark plus one light period equals 24 hours) is not necessary in order to demonstrate an endogenous rhythm. That is to say, there is no evidence that a "learning" or "memory" process is involved. For example, cells have been exposed to "non-diurnal" light and dark periods which together add up to greater or less than 24 hours, followed by conditions of either constant light or constant dark. An experiment of this sort is shown in Figure 3. In this experiment, cells were exposed to alternating light and dark periods of 7 hours each for about 100 hours. During this period the luminescence changes were quite evidently governed by these light and dark periods so that there was a maximum in luminescence every 14 hours. At the end of this treatment, some cells were placed in constant dim light and others in darkness. In both cases a diurnal rhythm with a period of approximately 24 hours was evident. The 14-hour cycle had not been "learned," even though it had been

possible to entrain the luminescence rhythm to the 14-hour cycle. A difference between those placed in darkness and those in dim light was that the amplitude of the rhythm in darkness progressively decreased as a result of the lack of light (see introduction).

Similar experiments have been carried out in which the alternating light and dark periods were 6 hours each, 8 hours each, and 16 hours each, giving cycles of 12, 16 and 32 hours, respectively. The results were similar to those shown in Figure 3. After about 100 hours of such a non-diurnal light-dark cycle the cells were placed in constant dim light and a rhythm of luminescence having a period close to 24 hours was evident.

Another series of experiments has shown that it is not necessary to pre-treat the cells with any sort of alternating light and dark periods in order to demonstrate endogenous diurnal rhythmicity. As mentioned previously, if cells are grown in continuous bright light (*ca.* 800–1500 foot-candles) there is no detectible rhythmicity. Cells maintained in this way for several months, or for as long as several years, have been found to exhibit a diurnal rhythmicity when they are placed in darkness (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a). The phase of the rhythm which is initiated when the cells are moved from bright light to darkness is independent of the solar time, and related only to the time at which the light-to-dark transition is made.

A similar result was obtained when cells which had been grown in bright light for almost one year were merely transferred to dim light. This experiment is

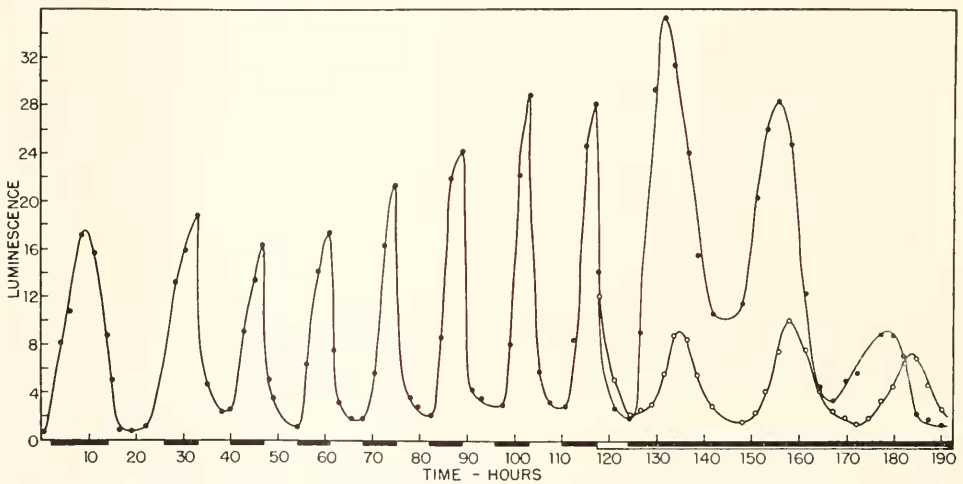

FIGURE 3. This illustrates the entrainment of the luminescence rhythm to a 14-hour cycle and the manifestation of an endogenous diurnal rhythm when the cells are placed in constant conditions subsequent to the treatment. Dark periods are indicated by black bars on the time axis. The cells were on an LD schedule previous to the time when the 14-hour cycle was started (at 26 hours). Light intensity throughout the 14-hour cycling was 800 foot-candles. At 117 hours some aliquots were removed from the dark and placed in constant light at 230 foot-candles. The luminescence changes in these cultures are shown by the circles. From 124 hours on, the other aliquots were left in the dark and the luminescence changes are plotted with solid dots. Temperature, 21° C.

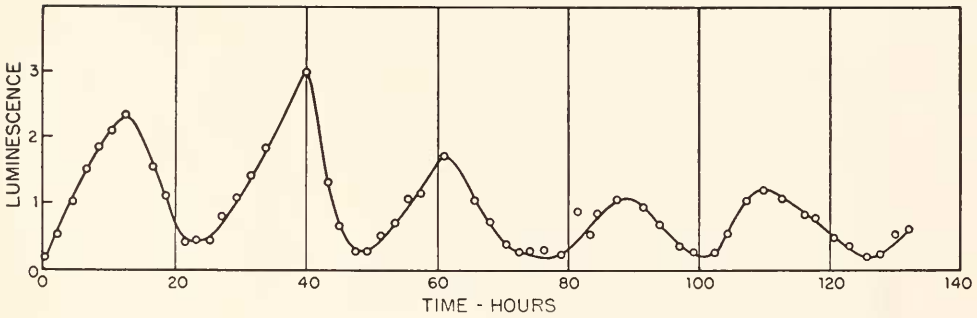

FIGURE 4. The initiation of an endogenous diurnal rhythm of luminescence by means of a one-step change in illumination. Cultures which had been grown in bright light for one year were moved from bright light (800 foot-candles) to dim light (90 foot-candles) at the time indicated on the graph as 0 hours. Luminescence measurements were made approximately every two hours thereafter. Temperature, 21.0° C. Average period, 24.5 hours.

illustrated in Figure 4. It differs from the previously mentioned experiment (in which cultures were moved from bright light to darkness) in that the amplitude does not decrease with time, since light is available for the nutrition of the cells. The precise phase relationship to the time of transfer from bright light is somewhat different, but here also it is not related to solar time.

Phase shift by light perturbation. It is clear from Figure 2 that the phase of the rhythm may readily be shifted by an appropriate manipulation of the light and dark periods to which the cells are exposed. It is not necessary, however, to expose the cells to a new light-dark cycle in order to reset the phase of the rhythm. A single exposure to a different light intensity can result in a stable phase shift. Pittendrigh and Bruce (1957) have discussed the significance of phase resetting of biological rhythms by single light perturbations. If rhythmicity results from an innate oscillatory mechanism characterized by its own natural period, and the phase (but not the period) is determined by the sequence of light and darkness, then it is to be expected that non-repeated light changes should suffice to change the phase. The perturbation therefore need not contain any information concerning period.

The experiment shown in Figure 4 illustrates phase setting by a single step-type light perturbation. The phase of the previously aperiodic cells was determined by the time at which the light intensity was changed. The shifting of phase in already rhythmic cultures is evident in the experiments shown in Figure 3. The entrainment of the rhythm to a 14-hour cycle may be explained by assuming that each transition, either from darkness to light or from light to darkness, serves to shift the phase, so that repetitive phase resetting occurs.

A phase shift in the *Gonyaulax* rhythm by single light perturbations has also been demonstrated in other ways. Figure 5 illustrates a shift in the phase of rhythmic cells which were given a single exposure to either bright light or darkness. The phase shift which results in such experiments has been found to be stable since, in experiments where measurements were continued for an additional 48 hours, the phase difference between the controls and the treated cells remained unchanged.

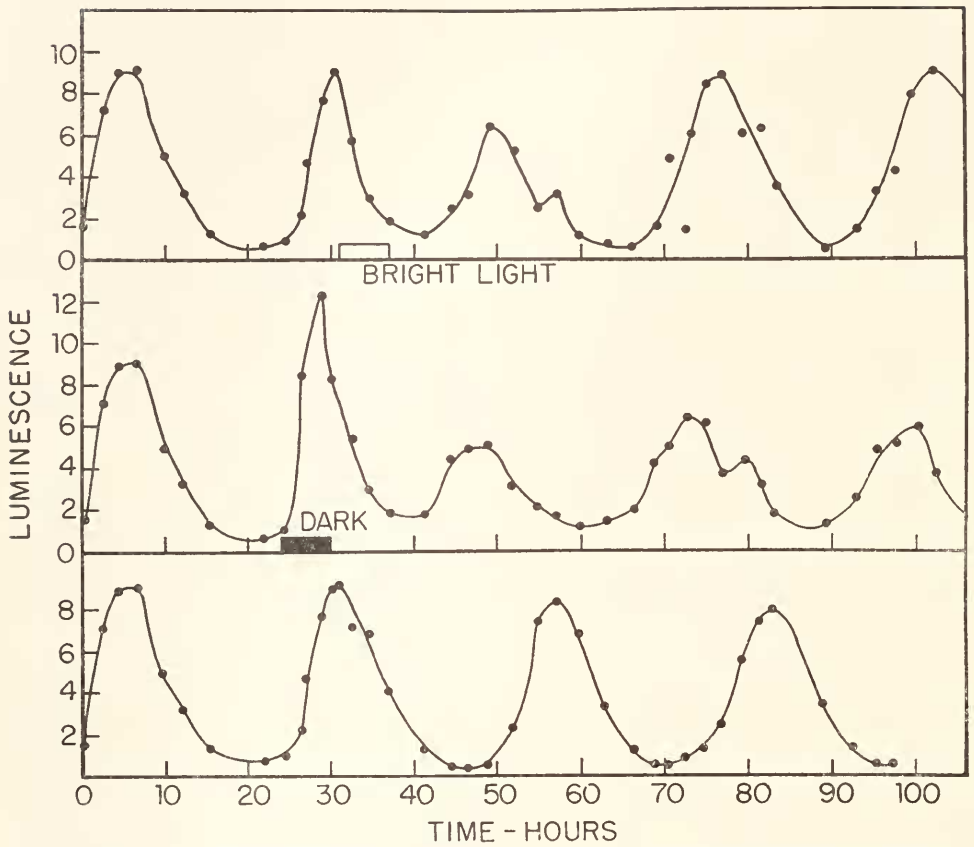

FIGURE 5. This experiment illustrates a phase shift in the rhythm following changes in light intensity. Cells previously kept under LD conditions were placed at constant temperature (23.5° C.) and constant light intensity (100 foot-candles) at the end of a 12-hour dark period. Two days later (zero time on the graph) measurements of luminescence were begun and the endogenous rhythm was apparent. Some cultures (upper curve) were transferred to bright light (1400 foot-candles) for a period of 6 hours and then returned to the previous condition (100 foot-candles). Other cultures (middle curve) were transferred to darkness for 6 hours and returned to dim light at 200 foot-candles. The time at which treatment was given is indicated by bars on the time axis. In both cases a marked phase shift in the rhythm is evident. The control (lower curve) was left in dim light all the while. Average period in control: 25.7 hours.

Figure 6 shows another technique which has been used in the study of phase shifting by single perturbations. Rhythmic cells were placed in the dark and, at a later time, received an exposure to light. Although the amplitude of the rhythm decreases over the next few days, the times at which maxima in luminescence occur are evident, so that the phase may be determined. The number of hours by which the phase is shifted would be expected to be some function of both the magnitude of the perturbation, and the time in the old cycle at which it is administered. The technique of interrupting darkness by light has been used to investigate these parameters.

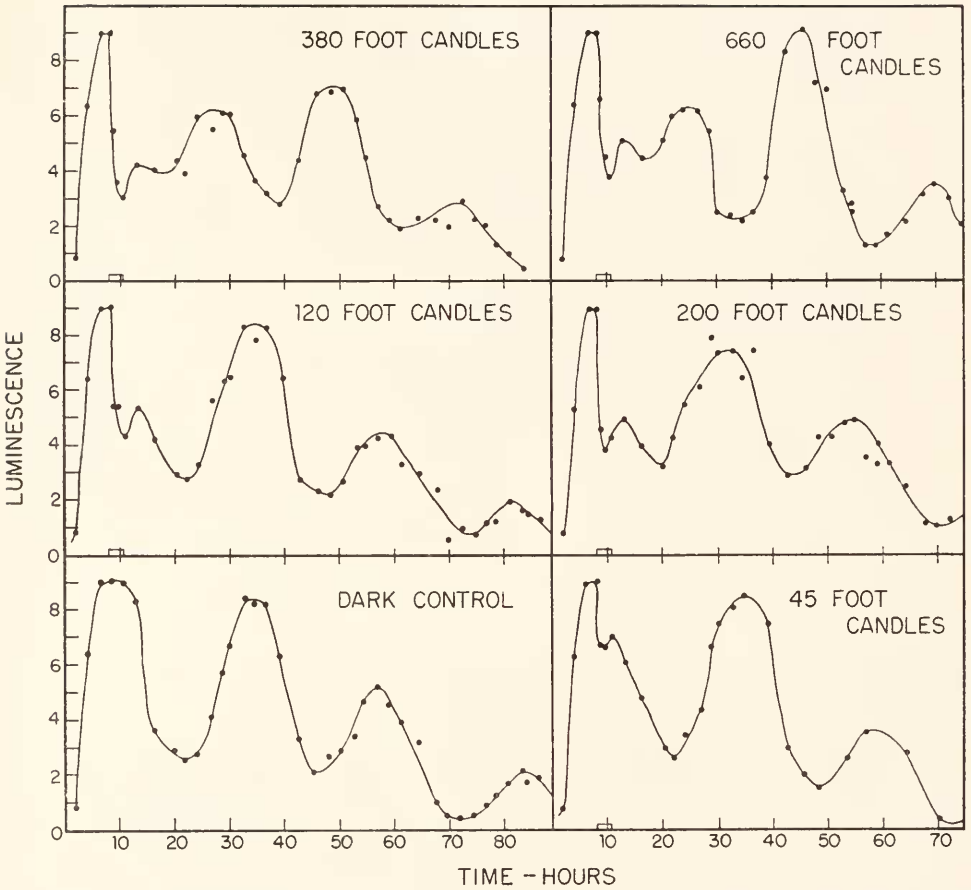

FIGURE 6. This illustrates phase shifting in a rhythmic culture by a single 2½-hour exposure to light, and the effect of intensity upon the magnitude of the phase shift. Prior to the time shown on the graph, all cultures were in LD conditions, and "two hours" on the time axis was the end of the last 12-hour light period. All cultures were put in the dark at that time and the control was left in the dark thereafter. The remaining cells were exposed to a 2½-hour illumination beginning 6 hours after the light-to-dark transition (indicated by the rectangle on the time axis). Following this 2½-hour illumination they were returned to darkness for the remaining time. The intensities used are shown in the figure. A 2½-hour exposure to 1400 foot-candles (not plotted) was found to be no more effective than the exposure to 660 foot-candles (Fig. 7). Temperature during experiment, 21° C.

The effect of varying the light intensity was determined in experiments such as the one shown in Figure 6. The amount of phase shift was found to increase with increasing light intensities, up to a "saturation" value of about 800 foot-candles. This relationship is illustrated in Figure 7, and the stability of the resetting is shown by plotting on the same graph the phase shift measured at each of the subsequent cycles. Several experiments of this sort have been carried out and the same type of relationship has been observed. The quantitative values obtained in separate experiments were somewhat different, however, and the reason for this variation has not been determined.

The magnitude of the perturbation may also be changed by varying the duration of light exposure. In an experiment similar to that shown in Figure 6, the duration instead of the intensity was varied. All exposures (at 800 foot-candles) were started simultaneously, six hours after the cells were placed in darkness. A longer exposure to such a light perturbation was found to be more effective than a shorter exposure. The amount of phase shift was found to be proportional to the duration of the exposure, up to a maximum phase shift of about 11½ hours, which was achieved with 2½ hours exposure. The relationship between phase shift and duration might be expected to be different, depending upon the time in the old cycle at which the perturbations were given, as discussed below. This aspect has not been studied, however.

The effect of varying the time in the cycle at which the perturbation is given has been studied by again using a procedure similar to that used in the experiments shown in Figure 6. Cells grown in LD conditions were transferred to a dark

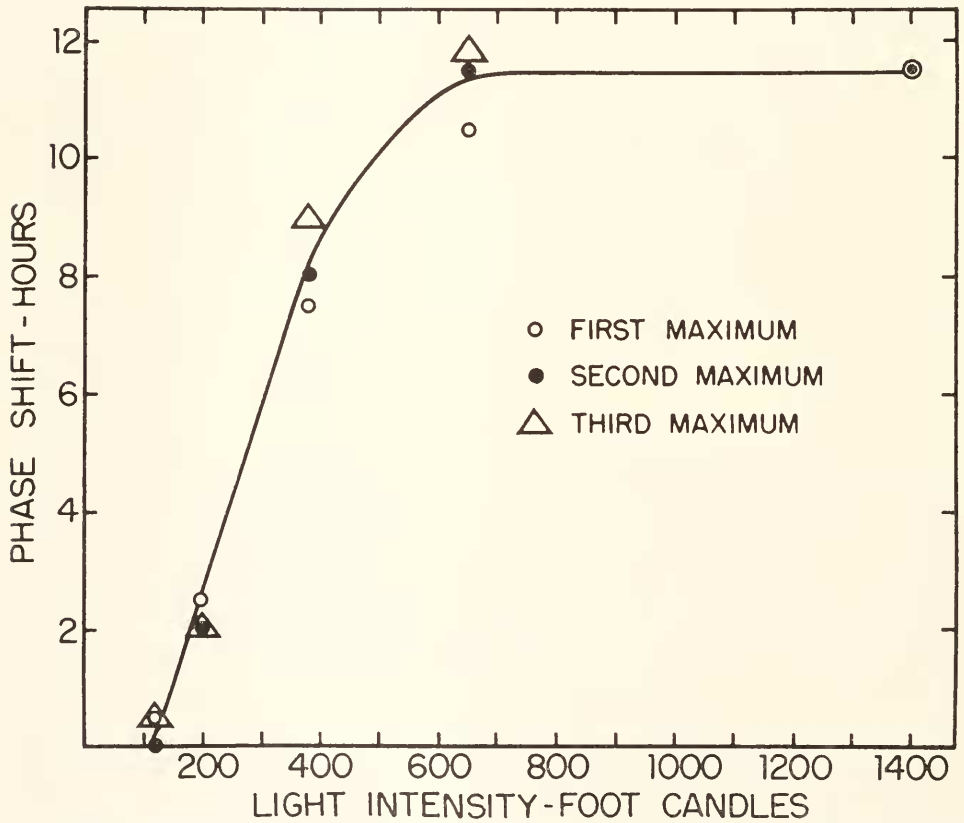

FIGURE 7. The relationship between the intensity of a single 2½-hour light perturbation and the number of hours by which the phase is shifted. Data taken from the experiments shown in Figure 6. Different symbols, as marked on the graph, give the phase difference between the control and the experimentals, measured at each of the three maxima in luminescence subsequent to the perturbation.

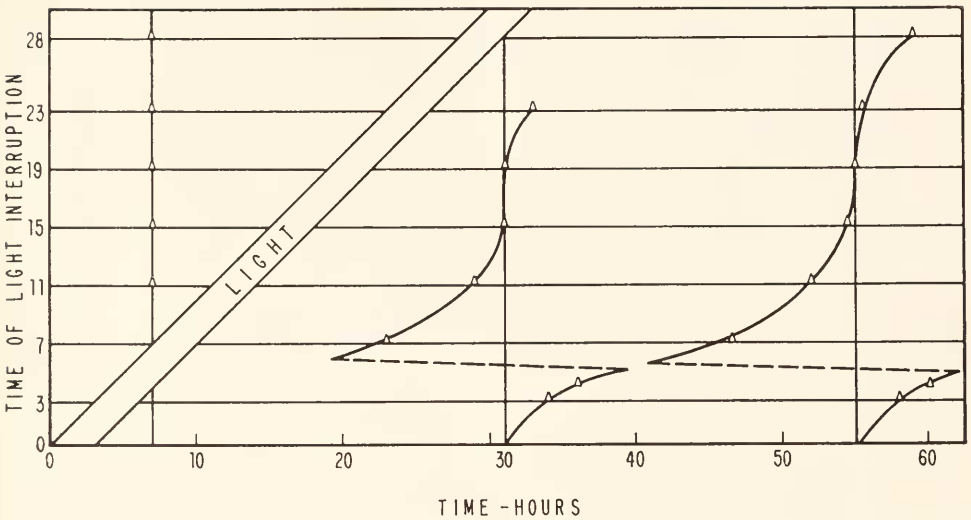

FIGURE 8. The effect of light perturbations (1400 foot-candles for 3 hours) given at different times during the cycle, upon the phase of the endogenous rhythm. Cells which had been kept under LD conditions were placed in the dark at zero time on the graph, which was the end of a 12-hour light period. The times at which the maxima in luminescence occurred in the control, which remained in the dark all the while, are indicated by vertical lines. In the experimentals, a triangular symbol shows a time at which a maximum in luminescence occurred, and thus represents phase. The experiments were carried out in a way similar to those illustrated in Figure 6. Each horizontal line represents a different experiment. For example, the line at 11 hours on the ordinate was an experiment in which a maximum in luminescence occurred at 7 hours. A light perturbation was begun at 11 hours and terminated at 14 hours. Maxima in luminescence occurred subsequently at 29½ hours and 52½ hours. The other experiments are represented in a similar way. The relationship between the time in the cycle at which the light perturbation was administered and the number of hours by which the phase was changed may be better visualized by rotating the figure by 90°.

chamber at the end of a light period. At regular time intervals thereafter, some of the cells were removed and exposed for three hours to light at an intensity of 1400 foot-candles, and then returned to darkness. Times at which exposures to light were made were selected so that the experiment served to scan somewhat more than a full 24-hour cycle. A control received no exposure to light, and the times at which maxima in luminescence occurred in this control are indicated by the vertical lines in Figure 8.

The results of the experiments are summarized in Figure 8. First of all, it may be noted that the new phase, following a light perturbation, was not directly related to the time at which the light perturbation was administered. That is, the maxima in luminescence did not occur at a fixed time interval following the light treatment. If that had been the case, the symbols indicating phase would fall along a line at 45°, parallel to the lines representing the times at which light exposures occurred. This latter type of result was obtained in experiments mentioned previously (Figure 4, for example) where a rhythm was initiated in an arrhythmic culture, and the phase was determined only by the time at which the light intensity was changed.

Secondly, it is apparent that the sensitivity to light perturbations was greater during the first 12 hours (Fig. 8) than during the second 12 hours of the cycle. During the first 12 hours a rather pronounced phase shift resulted, whereas during the second 12 hours there was little or no phase shift. In other longer term experiments it has been found that this variation in sensitivity continues in a rhythmic way. It may therefore be stated that, in general, the cells are maximally sensitive to a light perturbation at a time when luminescence is near maximum, and that this sensitivity declines to a minimum at a time when luminescence is minimum.

Finally, however, it may be noted from Figure 8 that a light exposure given before the maximum in luminescence results in a phase delay, so that the time between the light perturbation and the subsequent maximum in luminescence is greater than 24 hours. On the other hand, a light exposure given after the maximum in luminescence results in a phase advance, such that the next maximum in luminescence occurs in less than 24 hours. This difference is illustrated by the light perturbations which start at three hours and at seven hours in Figure 8.

Perturbation by mechanical stimulation. It is of interest to consider the nature of the cellular component or components which, being modified as a result of the light perturbation, result in the observed phase shift. If perturbation by means other than light also resulted in a change in the components of the rhythmic mechanism, then a phase shift would be similarly expected. It seemed possible that mechanical stimulation might be effective in this regard. Consequently, a perturbation experiment was carried out, in which air was bubbled through the cell suspensions instead of exposing the cells to light (Fig. 9). No phase shift occurred; the cells which had been stimulated retained the same phase as the unstimulated controls.

The experiment also shows that it is possible to modify the concentrations of compounds which are involved in the luminescence rhythm without having any effect upon the phase of the rhythmic mechanism itself. It was found previously (Hastings and Sweeney, 1957a) that the rhythm of luminescence involves a daily variation in the amount of extractable components of the luminescent system (luciferin and luciferase). Mechanical stimulation causes the luminescent reaction to occur, so that one would suppose that the concentrations of components in the luminescent system (and other biochemical systems coupled to it) might be changed. In fact, the apparent effect of stimulation is similar to the effect of light; the luminescence decreases to a low level in both cases. But since no phase shift occurred following stimulation, it does not seem likely that the luminescent system could be directly involved in the basic rhythmic mechanism, although it is clearly coupled to such a mechanism. Moreover, it is evident that there is no feedback from the luminescent system to the system controlling the phase of the rhythm. From previous evidence we had suggested that the luminescent system might itself constitute an autonomous chemical oscillation (Hastings and Sweeney, 1957b). The results described above, however, favor a hypothesis which proposes a basic mechanism of cellular rhythmicity to which various physiological and biochemical processes, such as luminescence or cell division (Sweeney and Hastings, 1957b; 1958), could be coupled.

Cellular interaction. Since all the experiments which have been described are carried out with large cell populations (4000–15,000 cells per tube), the ques-

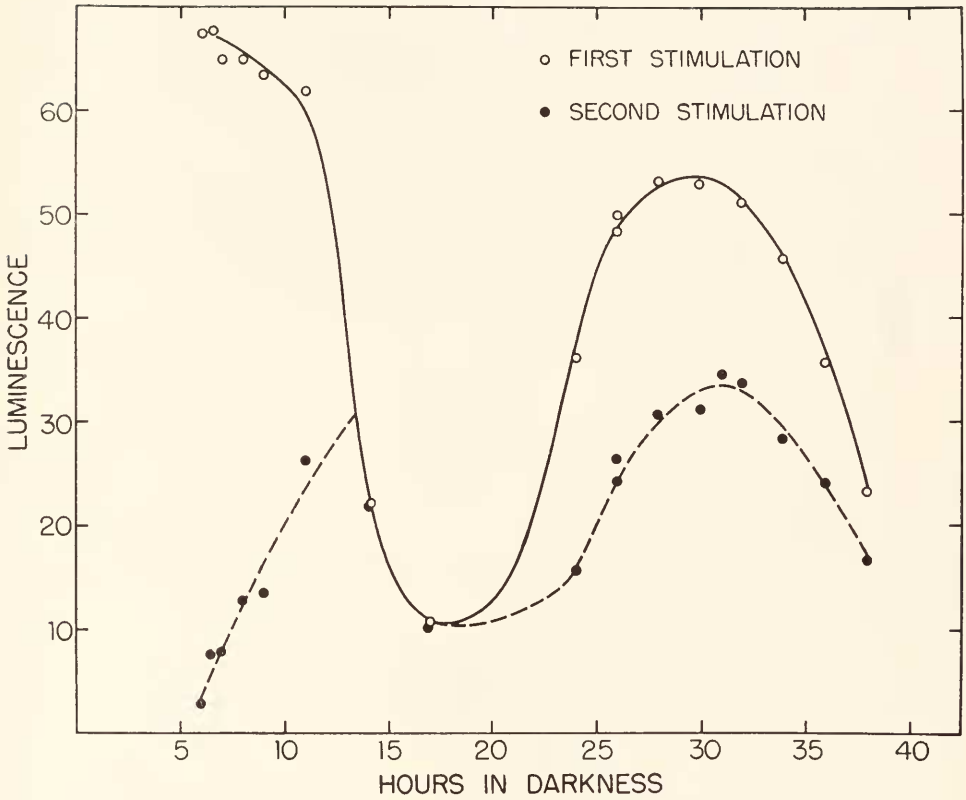

FIGURE 9. The effect of perturbation by mechanical stimulation upon the phase of the rhythm. Previous to the time shown, all cultures were in LD conditions, and zero time on the graph was the end of a light period. At this time all aliquots were placed in the dark. Six hours later a large number of aliquots were stimulated by bubbling air, but were not exposed to light. The luminescence changes of both these and the controls were determined through the subsequent maximum in luminescence. No significant change in the phase of the stimulated cultures was observed.

tion arises as to whether or not some cellular interaction might occur. Since the rhythmic mechanism involves fluctuations in the concentrations of chemical components within the cells, it is conceivable that certain diffusible compounds might escape into the medium, and that their concentrations might also fluctuate in a diurnal fashion. The importance of such a phenomenon would be evident if the supposed compound or compounds could function, as in a feedback mechanism, for stabilizing the frequency and/or phase of the rhythm. It is also possible that some other phenomenon, such as cellular motility, could be involved in such a feedback mechanism. This latter possibility seems unlikely, however, in view of the fact that mechanical stimulation, with its attendant violent motion and disturbance of cellular motility, did not result in a phase change.

An experiment in which this question was investigated is illustrated in Figure 10. Two cultures were maintained under LD conditions for several weeks with

their phases different by 5 hours. Samples were pipetted from each culture and moved to constant dim light at the end of a dark period. After each had been under constant conditions for several days (their phases still being different by

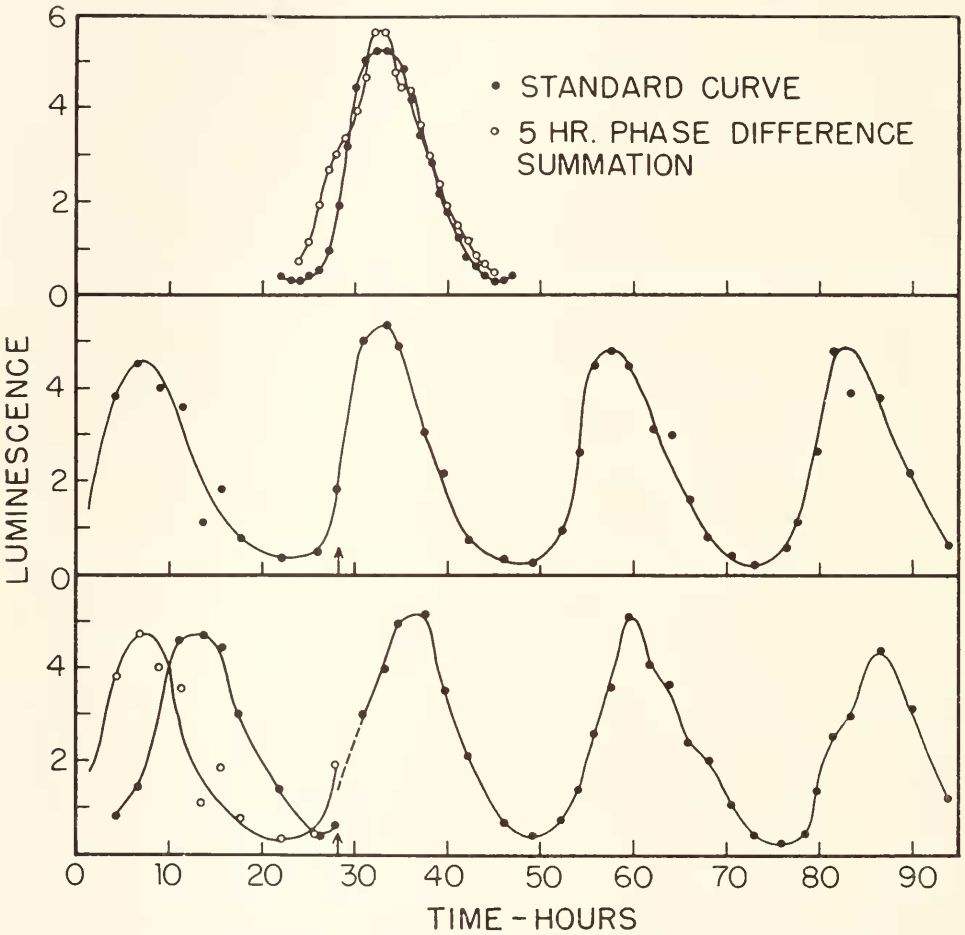

FIGURE 10. The effect of mixing two rhythmic cultures which were out of phase with one another. Cultures which had been in constant dim light for several days, having a 5-hour phase difference as shown (bottom curves), were mixed at the time indicated by the arrow. The rhythm continued, with a phase having its maximum at a time precisely halfway between the maxima of the two original cultures. The middle curve shows the result of mixing cultures having the same phase, done at the time indicated by the arrow. No change of phase was observed. The upper graph shows the result which would be theoretically expected upon mixing two cultures 5 hours out of phase, on the assumption that no interaction was involved. Two "standard" luminescence curves, which were measured from a culture which had not been mixed, were summated with a 5-hour phase difference. For purposes of comparison, the resultant curve is plotted on the graph along with the original standard curve, the latter having been displaced by 2½ hours on the time axis and normalized to the calculated curve. It may be seen that the shape of the calculated curve does not differ greatly from that of the original "standard" luminescence curve.

5 hours), the cultures were mixed in equal proportions, and the luminescence changes in the mixed cultures were measured.

If two typical curves showing the luminescence rhythm are summated, the phase of the two curves being different by five hours (75°), the resultant curve differs only slightly in shape from the original curves (Fig. 10, top). The maximum of the resultant curve lies precisely midway between the maxima of the two original curves.

In the actual mixing experiment, the maximum in luminescence of the mixed cultures occurred halfway between the maxima of the two separate unmixed cultures. Moreover, the shape of the curve from the mixed cultures was very similar to that which was obtained when the measured luminescence of the separate cultures was summated. The mixing experiment therefore indicates that no cellular interaction was involved.

Discussion

The subject of persistent endogenous rhythms has been recently reviewed by Harker (1958), Pittendrigh and Bruce (1957), and Bünning (1956). These reviewers, as well as other authors, have taken the view that the property of rhythmicity may be a nearly universal feature of organisms. This view is derived, largely, from the observation that endogenous rhythms are extremely widespread, having been reported from a large variety of both plants and animals. Furthermore, Pittendrigh and Bruce develop the generalization that most, if not all organisms can measure time; that they possess clocks. They consider that the basic mechanism evolved early, and that it has been retained in the course of evolution as a part of the adaptive organization of all organisms. Their use of the word "clock" refers to the basic mechanism involved in cellular rhythmicity, and the essential properties of this mechanism are considered to be similar in different organisms.

Pittendrigh and Bruce (1957) thus distinguish between the clock as the basic mechanism, and the persistent rhythms which are presumed to be controlled by the clock. Other authors (Brown, Hines, Webb and Fingerman, 1950; Stephens, 1957a; Harker, 1958) have similarly concluded that an overt persistent rhythm may be distinguished from an underlying mechanism, and our studies with *Gonyaulax* give support to this thesis. For example, since it was found that concentrations of compounds taking part in the luminescent reaction could be changed without shifting the phase of the rhythm, it is probable that the luminescence rhythm does not in itself constitute the basic mechanism. Furthermore, we have recently reported a persistent rhythm of cell division in *Gonyaulax* (Sweeney and Hastings, 1957b). The luminescence rhythm and the cell division rhythm have essentially identical properties. Moreover, we have not been able to demonstrate a phase shift in one rhythm which is not accompanied by a similar phase shift in the other rhythm. These findings give additional support to the hypothesis that one basic mechanism controls both rhythms.

The identity and physico-chemical nature of the presumed basic clock mechanism in persistent rhythms remains undefined. But if the properties of this basic mechanism in *Gonyaulax* may be deduced from the rhythm of luminescence, then it is evident that the mechanism possesses essential clock-like properties; the

period is not greatly affected by environmental factors, but the phase is labile to resetting by the appropriate external changes. We may note, in addition, that light emission in *Gonyaulax* is clocked so that it is maximal during the night phase, when it is visible; and without environmental inhibition, luminescence is minimal during the day phase. However, since the possible utility of the light emission is not known, the functional significance of clocked luminescence is not apparent.

Many of the characteristics of the rhythm of luminescence which we have described are similar to the characteristics of persistent rhythms in a variety of other organisms, ranging from other unicellular forms to mammals. The comparisons outlined below do not pretend to be complete, but they serve to illustrate the point. The remarkable similarities found support the view of Pittendrigh and Bruce (1957), that the basic mechanism involved in rhythmicity is the same in all organisms.

Practically all the persistent diurnal rhythms described have natural periods which are close to but different from 24 hours. This includes rhythms in *Drosophila* (Pittendrigh, 1954), *Uca* (Webb, Brown and Sandeen, 1954), *Oedogonium* (Bühnemann, 1955a), *Euglena* (Bruce and Pittendrigh, 1956), and many others. The natural period may range, in different organisms, from about 21 to 27 hours. In fact, significant differences in the natural periods in different individual mice are well documented (Pittendrigh and Bruce, 1957).

Studies of rhythms in a variety of organisms, including the bee (Wahl, 1932), *Uca* (Brown and Webb, 1948), *Avena* (Ball and Dyke, 1954), *Drosophila* (Pittendrigh, 1954), and *Euglena* (Bruce and Pittendrigh, 1956), have shown that in each case the period is nearly the same at temperatures which differ by 15° C., or more. It is interesting to note that the effect of temperature upon the period of the *Gonyaulax* rhythm is similar to that reported by Bühnemann (1955b) for the rhythm of sporulation in *Oedogonium,* in that the apparent Q_{10} for both is less than 1.0. Two cases may therefore be interpreted as the result of an over-compensation in the mechanism responsible for temperature independence (Hastings and Sweeney, 1957b).

Only a few experiments have been specifically designed to detect the effect of different light intensities upon the natural period of persistent rhythms. In those cases which have been reported (see Harker, 1958), the natural period has been found to change no more than an hour or two under different light intensities.

The entrainment of rhythms to periods different from 24 hours has been reported in several organisms, including *Euglena* (Bruce and Pittendrigh, 1956) and *Oedogonium* (Bühnemann, 1955a). In these and other cases, as in *Gonyaulax*, the rhythms return to the characteristic natural period when the organisms are returned to constant conditions.

On the other hand, several experiments have been reported in which rhythmic organisms still continue to show a 24-hour rhythm while being subjected to light-dark cycles which differ from 24 hours. For example, Webb (1950) found that the period of the *Uca* rhythm was not changed while the organisms were subjected to light (95 foot-candles) and dark periods of 16 hours each, and Tribukait (1954) found that entrainment to an imposed light-dark cycle occurred in the mouse only so long as the imposed cycles did not differ greatly from the natural period.

Studies with *Gonyaulax* suggest a possible reason for the lack of apparent entrainment in experiments such as those cited above: the light intensities used may not have been sufficiently bright. In *Gonyaulax,* the luminescence rhythm may be entrained to periods which differ greatly from the natural period. Our interpretation of this entrainment is that repetitive phase resetting results in a period corresponding to the imposed schedule. The importance of light intensity as a parameter in phase shifting by single light perturbations has been documented in experiments with *Gonyaulax.* That it is equally important in entrainment has been shown in an experiment with *Gonyaulax* described elsewhere (Hastings and Sweeney, 1958), in which it was found that entrainment occurred at a light intensity of 800 foot-candles, but not at 200 foot-candles.

Entrainment of rhythms to imposed cycles which are only slightly longer or shorter than the natural period has been discussed by Pittendrigh and Bruce (1957). Their interpretation suggests that the mechanism may be different from that involved in entrainment to cycles differing greatly from the natural period.

The role of 24-hour light-dark cycles in establishing the phase of diurnal rhythms has long been recognized, and experiments with many organisms have demonstrated that, as in *Gonyaulax,* the phase shifts in response to a new light-dark cycle which is out of phase with solar night and day. The fact that the light intensity used in such experiments is of importance has been shown by Brown, Fingerman and Hines (1954).

That non-repeated light perturbations are capable of establishing or changing the phase of a persistent rhythm has been stated as an important generalization only in recent years (Pittendrigh and Bruce, 1957), although some previous studies (Kalmus, 1940; Webb, 1950) do provide examples of the phenomenon. The phenomenon provides another analogy between the characteristics of persistent rhythms and the known properties of physical oscillators. It is well known that a single disturbance or perturbation applied to an oscillating system will quite generally shift its phase without any modification to the period, and the behavior of a simple pendulum is a good example. Pittendrigh and Bruce (1957) have found phase shifts following single light perturbations in persistent rhythms of *Euglena* and *Drosophila,* and the rhythm in *Gonyaulax* provides another example of the phenomenon.

Detailed studies on the effect of the duration and intensity of single perturbations have not yet been reported in other organisms, but it appears that the nature of the phase shift in *Gonyaulax* may differ in one respect from that reported for *Drosophila* (Pittendrigh and Bruce, 1957). Following a single light perturbation in *Drosophila* there may occur "transients," so that the phase is not reset immediately but comes to its stable position only after several cycles. In *Gonyaulax,* on the other hand, phase has been found to be reset immediately. The reason for this difference is not known, but it may be related to the relative complexity of the organisms involved.

With respect to the phenomenon of phase shifting, Bruce and Pittendrigh (1957) have discussed whether the resetting signal is the step-up in light intensity (dawn) or the step-down in light intensity (dusk). Several experiments with *Gonyaulax* have adequately illustrated that the phase is labile to both, so that

neither event may be said to be the timing cue to the exclusion of the other. For example, the experiments shown in Figure 3 illustrate both a light-to-dark transition followed by constant darkness, and a dark-to-light transition followed by constant light. In both cases, the last transition resulted in a phase shift.

The action spectrum for shifting the phase of the luminescence rhythm by a single light perturbation shows relatively sharp maxima in effectiveness at 475 mμ and 650 mμ (Hastings and Sweeney, unpublished). The red maximum, in particular, suggests that chlorophyll acts as a photosensitizer for phase shifting. Since the effects of single light perturbations are essentially the same in plants and animals, we may conclude that in *Gonyaulax* the photosensitizers involved in phase determination are not a part of the basic mechanism of rhythmicity. In animals, also, the photoreceptor pigments of the eye are not a part of the basic mechanism, although they function in phase determination by light. Whitaker (1940) reported that blinded mice possess a natural period of about 24 hours in their activity rhythm, but that the rhythm could not be entrained by 24-hour light-dark cycles to correspond with solar night and day, as in normal mice.

It is known that temperature changes (Pittendrigh, 1954; Stephens, 1957a), and perhaps certain other factors (Harker, 1958) may also serve to establish or reset phase. There is no report, however, that mechanical disturbances can be effective in other organisms in this regard.

The possibility that individuals in a population may entrain each other was suggested by Pittendrigh and Bruce (1957). However, Stephens (1957b) was unable to demonstrate any significant phase modification in individual fiddler crabs when they were placed together with crabs possessing a different phase. A similar result was found in the present studies with *Gonyaulax*.

It is of interest to note that the shape of the luminescence curve obtained in experiments where *Gonyaulax* cultures possessing different phases were mixed is not greatly different from that for the unmixed cultures. Indeed, as already pointed out, this is the expected result of adding two luminescence curves which are five hours out of phase with one another. Thus, a population composed of cells having at least two different phases is difficult to distinguish from the usual experimental populations, in which we have assumed that all cells possess the same phase. This experiment serves to caution us. In a biological rhythm having a sinusoidal shape, measurements from populations may not accurately represent the behavior of individual cells.

We do not know how the luminescence of the individual *Gonyaulax* cell at different times in the cycle compares with that measured in a population. The question is an important one, and there are several possibilities which, in the absence of any relevant data, need not be discussed here. This problem is being investigated utilizing measurements of the rhythm of cell division, where the performance of an individual cell may be repeatedly and relatively easily scored.

Although several suggestions have been made concerning the physico-chemical nature of the basic mechanism involved in persistent diurnal rhythmicity (Pittendrigh and Bruce, 1957; Hastings and Sweeney, 1958), none has received any substantial support. It is hoped that information concerning the extent and kind of biochemical changes associated with the rhythms will be of value in understanding this basic problem. Studies of this nature are in progress with *Gonyaulax*.

Summary

1. The characteristics of a persistent diurnal rhythm of luminescence in the dinoflagellate *Gonyaulax polyedra* are described.

2. The light emission upon stimulation, from cultures which are kept in alternating light and dark periods of 12 hours each (= LD), is 40 to 60 times greater during the dark period than during the light period. If LD cultures are placed in continuous dim light (100 foot-candles) a diurnal rhythm of luminescence persists. If LD cultures are placed in continuous bright light (> 1500 foot-candles) the rhythm is damped, and no fluctuations occur in the amount of light emitted.

3. The occurrence of rhythmicity is not dependent upon prior exposure to LD conditions. Cultures which have been grown in bright light for as long as one year show a diurnal rhythm when placed in constant dim light or darkness. Cultures kept in alternating light and dark cycles which are greater or less than 24 hours similarly show a diurnal rhythm when returned to constant dim light or darkness. "Training" or "memory" is therefore not involved.

4. The rhythm can be entrained by light-dark cycles which are different from 24 hours. The period of the luminescence rhythm corresponds to light-dark cycles which have periods ranging between 12 and 32 hours.

5. The period of the rhythm is always close to 24 hours when the cells are kept under constant conditions, but it varies slightly depending upon the temperature and light intensity.

6. The phase of the rhythm under constant conditions is related to the time at which the previous light and dark periods occurred. Moreover, the phase may be shifted by interposing a non-repeated exposure to a different light intensity. The number of hours by which the phase is shifted in such an experiment is dependent upon the intensity and duration of the light treatment, and the time in the cycle when it is administered.

7. Exhaustive mechanical stimulation does not alter the phase of the rhythm.

8. When cultures having different phases were mixed, no evidence was found which would indicate that there was any interaction between them.

9. The evidence presented indicates that the diurnal rhythmicity is the consequence of a basic oscillatory mechanism which is inherent to the cell.

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